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Acute Toxicity of 33 Herbicides to the Green Alga *Chlorella pyrenoidosa*

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In the present world, environmental problems are multiple and complex, especially those arising from the disposal of identify and assess the toxicity of such substances. Assessment of human exposure to pesticides and other toxicants through biological monitoring offers one means to evaluate the magnitude of potential health risk of these chemicals (Bhatnagar et al. 1992; Ghosp et al. 1997). Herbicides play an important role in agricultural practices, particularly for cereals (Berard 1994). The increased usage of herbicides has elicited extensive research into herbicide effects on non target organisms such as algae. Their potential effect on the aquatic primary producers is particularly important, and has to be studied in ecotoxicological experiments (Berard 1996). Algae play an important role in the primary production of the aquatic ecosystem. Herbicides can affect the structure and function of aquatic communities through alterring species composition of an algal community. Algae have been considered to be indicators of the bioactivity of industrial wastes. Unicellular algae vary in their response to a variety of toxicants (Tadros et al. 1994). Little is known, however, about the toxicity of new herbicides to freshwater green algae (Fargasova 1996). The work reported here was done to examine the effect of 33 herbicides on the green alga *Chlorella pyrenoidosa.*

MATERIALS AND METHODS

The green alga *Chlorella pyrenoidosa* was used as the test organism and was obtained from Institute of Wuhan Hydrobiology, the Chinese Academic of Science. Cells of *Chlorella pyrenoidosa* were propagated photoautotrophically in a 250 mL Erlenmeyer flask containing 100 mL liquid HB-4 medium (Li 1959) and kept on a rotator shaker (100 rpm) at 25℃, and illuminated with cool-white fluorescent lights at a continuous light intensity of 5000 lux/cm^2. The culture medium was sterilized at 121℃, 1.05 kg cm^{-2} for 30 min (Kong et al. 1999). For cell experiments, 15 mL aliquots of the HB-4 medium containing green algal cells (about 6×10^5 cells/mL (initial cell concentration)) were distributed to sterile 50 mL Erlenmeyer flasks. The medium of *Chlorella pyrenoidosa* were then treated with various herbicides concentrations ranging from zero to 50 mg/L, and incubated for 96 hr on an orbital shaker (100 rpm) at a temperature of 25℃ and a continuous light intensity of 5000 lux/cm^2. Cell counts were correlated with

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absorbance over time for 96 hr on a Shimadzu UV-2401PC spectrophotometer. The most suitable wavelength to use for monitoring culture growth was 680 nm. Each herbicide concentration was replicated three times. Appropriate control systems containing no herbicide were included in each experiment. Control and treated cultures grew under the same conditions of temperature, photoperiod and shaking of the stock cultures. In each experiment, percent inhibition values, relative to growth in control systems, were calculated using spectrophotometric data. The EC_{50} values (herbicide concentration required to cause a 50% reduction in growth) were calculated using linear regression analysis of transformed herbicide concentration as natural logarithm data versus percent inhibition (Grossmann et al. 1992). All correlation coefficients were >0.85. All of tested herbicides were purchased from People's Republic of China and their chemical classes and influenced mechanisms (Retzinger et al. 1997; Su 1998) are shown in Table 1. The tested herbicides were dissolved by acetone, methanol or distilled water.

RESULTS AND DISCUSSION

Cells of *Chlorella pyrenoidosa* were counted under a microscope. The count of algal cells is well proportioned to the absorbance at 680 nm. The linear regression equation is $C=0.4912+34.7725 \times A$, coefficient of correlation $r=0.9975$, significance level $P=0.0001$. Meanwhile, the reduction in conductivity is inversely proportioned to the increase in growth. The linear regression equation is $C=-0.7501+0.1744 \times \triangle EC$, coefficient of correlation $r=0.9884$, significance level $P=0.0002$. Hence, growth of algal cells was calculated indirectly using spectrophotometric data.

Acute toxicity of 33 herbicides to the green alga *Chlorella pyrenoidosa* is shown in Table 2. The 96 hr EC_{50} values of herbicides which block the *de novo* sysnthesis of fatty acids by inhibiting the activity of acetyl-CoA carboxylase (ACCase) (Saenz et al. 1997) varied around 0.7~15 mg/L (10^{-5}-10^{-6} M), fluazifop-p, quizalofop-p and haloxyfop-R were about 10^{-5} M level, fenoxaprop and diclofop-p were about 10^{-6} M level. The 96 hr EC_{50} values of acetolactate synthase (ALS) inhibitors which block the biosynthesis of the branched-chain amino acids leucine, isoleucine, and valine varied around 0.1~26 mg/L (10^{-5}-10^{-7} M), nicosulfuron, ethametsulfuron, bispyribac+spreader were about 10^{-6} M level and cyclosulfamuron achieved 10^{-7} M level. The average acute toxicity of ALS inhibiting-herbicides to the green alga *Chlorella pyrenoidosa* was lower than that of ACCase inhibiting herbicides. The 96 hr EC_{50} values of protoporphyrinogen oxidase (Protox) inhibiting herbicides such as oxyfluorfen and oxadiargyl varied around 4~5 mg/L (10^{-5} M). Protox inhibitors leads to the accumulation of its substrate protoporphyrinogen, which is readily oxidized to proto IX by oxidative enzymes. Proto IX is an effective photosensitizer, and in the light, it transfers absorbed energy to molecular oxygen to form singlet oxygen. The singlet oxygen peroxidizes lipids leading to the destruction of cellular membranes (Moreland DE 1999). The 96 hr EC_{50} values of herbicides, such as pretilachlor

Table. 1 Selected herbicides and chemical classes

Herbicides	Chemical class	Influenced mechanisms	Formuations
Diclofop-p	Aryloxyphenox propionates	Acetyl-CoA carboxylase (ACCase)	99.5% TC[a]
Quizalofop-p			5% EC[b]
Haloxyfop-R			10.8% EC
Fenoxaprop			6.9% EC
Fluazifop-p			53% EC
Nicosulfuron	Sulfonylureas	Acetolactate synthase (ALS)	4% SC[c]
Metsulfron methyl			90% TC
Cyclosulfamuron			10% WP[d]
Tribenuron			95% TC
Ethametsulfuron			25% WP
Bispyribac	Dimethoxypyrimi-dinylsalicylic acids		98% TC
Bispyribac+spreader			10% SC
Anilofos	Organophosphorus	Unknown	30% EC
Pendimethalin	Dinitrophenols	Microtubue process	33% EC
Cinmethylin	Benzylether	Mitotic process	10% EC
Pretilachlor	Chloroacetamides	Cell division	93% TC
Butachlor			90% TC
Mefenacet			95% TC
Atrazine	Triazines	Photosynthetic process	38% SC
Simazine			92% TC
Ametryne			92% TC
Prometryne			77% TC
Isoproturon	Ureas		95% TC
Chlorotoluron			95% TC
Diuron			50% WP
Paraquat	Bipyridyliums		20% SL[e]
Fluroxypyr	Pyridnecarboxylic	Hormones synthesis	11% EC
Quinclorac	Quinoline acids		90% TC
MCPA	Phenoxycarboxylic acids		10% SL
Benazolin- ethyl	Others		50% SC
Oxyfluorfen	Diphenylethers	Protoporphyrin-ogn oxidase (Protox)	81% TC
Oxadiargyl	Oxadiazole		80% SL
Glyphosate	Glycines	EPSP synthase	95% TC
Molinate	Thiocarbamates	Lipid synthesis	96% EC

[a]TC (technical product); [b]EC (emulsible concentrate);[c]SC (suspension concentrate); [d]WP (wettable powder) [e];SL (soluble concentrate)

Table 2. Dose response relationship of 33 herbicides to *Chlorella pyrenoidosa*

Herbicides	Regression Equation	S L[a]	CC[b]	EC_{50} (mg/L)	EC_{50} (M)
Diclofop-p	P[c]=54.655+12.534lnC[d]	0.057	0.9435	0.690	2.02×10^{-6}
Fluazifop-p	P=-5.356+20.122lnC	0.048	0.9517	15.660	4.78×10^{-5}
Quizalofop-p	P=2.702+27.376lnC	0.074	0.9261	5.628	1.53×10^{-5}
Haloxyfop-R	P=11.145+23.187lnC	0.020	0.9744	5.340	1.23×10^{-5}
Fenoxaprop	P=48.587+23.730lnC	0.054	0.9460	1.001	2.76×10^{-6}
Molinate	P=16.172+20.921lnC	0.133	0.8667	5.038	2.69×10^{-5}
Bispyribac	P=-59.448+35.841lnC	0.028	0.9719	21.194	4.68×10^{-5}
Bispyribac+[e]	P=23.450+26.110lnC	0.050	0.9500	2.760	6.42×10^{-6}
Cyclosulfa-muron	P=69.2338+9.825lnC	0.011	0.9892	0.141	3.35×10^{-7}
Tribenuron	P=-7.010+11.387lnC	0.115	0.8854	26.544	6.71×10^{-5}
Ethametsul-furon	P=42.975+11.653lnC	0.001	0.9933	1.827	4.45×10^{-6}
Nicosulfuron	P=36.334+17.340lnC	0.030	0.9697	2.200	5.36×10^{-6}
Metsulfron methyl	P=-11.34+23.107lnC	0.028	0.9746	14.220	3.73×10^{-5}
Butachlor	P=28.752+16.468lnC	0.002	0.9846	3.630	1.16×10^{-5}
Pretilachlor	P=36.846+14.010lnC	0.040	0.9244	2.560	8.21×10^{-6}
Mefenacet	P=69.548+4.111lnC	0.014	0.9860	8.6×10^{-3}	2.88×10^{-8}
Cinmethylin	P=83.054+4.295lnC	0.020	0.9796	4.5×10^{-4}	1.64×10^{-9}
Paraquat	P=101.026+5.531lnC	0.007	0.9934	1.0×10^{-4}	5.36×10^{-10}
Ametryn	P=94.716+5.447lnC	0.082	0.9182	3×10^{-4}	1.41×10^{-9}
Atrazine	P=85.324+17.762lnC	0.119	0.8803	0.145	6.72×10^{-7}
Simazine	P=83.732+13.523lnC	0.003	0.9655	0.082	4.09×10^{-7}
Chlorotolurn	P=2.702+27.376lnC	0.040	0.9566	1.490	7.01×10^{-7}
Isoproturon	P=105.03+10.412lnC	0.058	0.9423	0.005	2.42×10^{-8}
Diuron	P=116.440+10.904lnC	0.000	0.9980	1.3×10^{-3}	5.59×10^{-9}
Prometryn	P=89.1012+8.824lnC	0.070	0.9299	0.012	4.93×10^{-8}
Pendimethan	P=61.288+12.125lnC	0.120	0.8797	0.394	1.40×10^{-6}
Anilofos	P=25.685+12.215lnC	0.115	0.8842	7.320	1.99×10^{-5}
MCPA	P=25.688+7.870lnC	0.014	0.9856	21.960	7.30×10^{-5}
Benazolin-ethyl	P=19.113+8.537lnC	0.002	0.9828	37.260	1.15×10^{-4}
Fluroxypyr	P=35.516+13.013lnC	0.084	0.9152	3.044	8.24×10^{-6}
Quinclorac	P=48.708+5.464lnC	0.101	0.8983	1.267	5.23×10^{-6}
Oxadiargyl	P=-0.325+18.132lnC	0.014	0.9651	5.375	1.58×10^{-5}
Oxyfluorfen	P=45.903+2.929lnC	0.004	0.9531	4.008	1.11×10^{-5}
Glyphosate	P=31.361+14.769lnC	0.072	0.8877	3.530	2.09×10^{-5}

[a]SL (significance level); [b]CC (coefficient correlcation); [c]P(percent inhibition)
[d]C(herbicide concentration) ; [e]bispyribac+(bispyribac+spreader)

and butachlor which influence cell division varied around 2~4 mg/L (10^{-5} M). Mitotic Process inhibitor--cinmethylin arrived at 10^{-9} M level. Its acute toxicity was higher than that of ALS inhibitors, ACCase inhibitors or Protox inhibitors to the green alga *Chlorella pyrenoidosa*. The 96 hr EC_{50} values of pendimethalin which influenced the microtubule mrocess was 0.4 mg/L (1.4×10^{-6} M). Auxin herbicides such as quinclorac, fluroxypyr varied around 1~3 mg/L (10^{-6} M level), MCPA and benazolin-ethyl varied around 20~40 mg/L (10^{-5} M level). Auxin herbicides stimulate ethylene biosynthesis by inducing the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. In susceptible dicots, increased levels of ethylene trigger an accumulation of abscisic acid (ABA). In susceptible grasses, the levels of tissue cyanide (HCN), a co-product formed during ethylene biosynthesis, increased. These increases in ethylene, ABA, and HCN cause epinasty of leaves, growth retardation, and senescence. However, not all researchers are in agreement about the association between plant sensitivity to the auxin type herbicides and the increase in ethylene production (Moreland 1999). Their acute toxicity to the green alga *Chlorella pyrenoidosa* were lower than others. The 96 hr EC_{50} values of 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSP synthase) inhibitors--glyphosate was 3 mg/L (2.09×10^{-5} M). It causes the concentration of glyoxylate to elevate which inhibits RuBP carboxylase, the first enzyme involved incarbon fixation. The 96 hr EC_{50} values of the photosynthesis-inhibiting herbicides was the lowest among of tested herbicides. The 96 hr EC_{50} values of chlorotoluron was 1 mg/L, atrazine 0.1 mg/L , prometryne and simazine 10^{-2} mg/L, isoproturon and diuron 10^{-3} mg/L, ametryne and paraquat 10^{-4} mg/L level, most of their molar concentration was 10^{-7}~10^{-9} M, neared or arrived at 10^{-9} M level. The acute toxicity of this type of herbicide to the green alga *Chlorella pyrenoidosa* was the highest among all of tested herbicides.

Electrical conductivity of algal medium was assayed. The relationship between cell density and the conductivity decrease was better in the control algal medium which contained no herbicides , but there was no relativity in the treatment which had the added different concentration of herbicides. Tested herbicides and organic solvents may have influence over algal cell uptaking inorganic mineral ion in medium, thus, it may be not fit for indicating algal growth amounts using the conductivity decrease of algal medium, although some reports (Grossmann 1992) thought this was true.

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